Small molecule MYC inhibitor conjugated to integrin-targeted nanoparticles extends survival in a mouse model of disseminated multiple myeloma

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Running title: Anti -MYC prodrug nanotherapy for myeloma

Key Words: cMYC, multiple myeloma, transcription factors, nanomedicine, phospholipid prodrug, nanoparticles, drug delivery
Financial Support: This work was supported by the Department of Defense Grant # CA100623 (M.H. Tomasson and G.M. Lanza). Further partial support for this work was derived from NIH HL113392 (G.M. Lanza), CA154737 (G.M. Lanza, K.N. Weilbaecher, D. Pan).

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Conflict of Interest Statement: The University of Pittsburgh (E.V. Prochownik) and Washington University (G.M. Lanza, D. Pan) have intellectual property interests related to aspects of the technologies described.

Abstract 238

Word Count: 4980

No. of figures: 5

References: 38
**Abbreviations:**

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<tr>
<td>°C</td>
<td>Degree Centigrade</td>
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<tr>
<td>bHLHZIP</td>
<td>Basic Helix loop Helix loop Zipper</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CFDD</td>
<td>Contact Facilitated Drug Delivery</td>
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<tr>
<td>D</td>
<td>Drug</td>
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<td>DCC</td>
<td>dicyclohexyl cardiimide</td>
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<tr>
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<tr>
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<td>Manganese</td>
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MTT     (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
n     nano
ND     No Drug
nm     nano meter
NP     Nanoparticles
NT     Non-targeted
p     pico
P/S     Penicillin/ Streptomycin
PAz     1-palmitoyl-2-azelaoyl
PBS     Phosphate Buffered Saline
PE     Phycoerythrin
PFC     Perfluorocarbon
PFOB    Perfluorooctylbromide
PLA-2    phospholipase 2
SD     Standard Deviation
SDS-PAGE     sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sn 2     bimolecular nucleophilic substitution
T     Targeted
v/v     volume / volume
VCAM-1     Vascular Cell Adhesion Protein 1
VLA-4    Very Late Antigen-4
w/v     weight/volume
α     alpha
β     beta
μ     micro
ABSTRACT

Multiple myeloma (MM) pathogenesis is driven by the MYC oncoprotein, its dimerization with MAX, and the binding of this heterodimer to E-Boxes in the vicinity of target genes. The systemic utility of potent small molecule inhibitors of MYC-MAX dimerization was limited by poor bioavailability, rapid metabolism, and inadequate target site penetration. We hypothesized that new lipid-based MYC-MAX dimerization inhibitor prodrugs delivered via integrin-targeted nanoparticles would overcome prior shortcomings of MYC inhibitor approaches and prolong survival in a mouse model of cancer. An Sn 2 lipase-labile prodrug inhibitor of MYC-MAX dimerization (MI1-PD) was developed and decreased cell proliferation and induced apoptosis in cultured MM cell lines alone (p<0.05) and when incorporated into integrin-targeted lipid-encapsulated nanoparticles (p<0.05). Binding and efficacy of nanoparticles closely correlated with integrin expression of the target MM cells. Using a KaLwRij metastatic MM mouse model, VLA-4 targeted nanoparticles (20nm and 200nm) incorporating MI1-PD (D) NPs conferred significant survival benefits compared to respective NP controls, targeted (T) no-drug (ND) and untargeted (NT) control nanoparticles (T/D 200: 46 days vs. NT/ND: 28 days, p<0.05 and T/D 20: 52 days vs. NT/ND: 29 days, p=0.001). The smaller particles performed the better of the two sizes. Neither MI1 nor MI1-PD provided survival benefit when administered systemically as free compounds. These results demonstrate for the first time that a small molecule inhibitor of the MYC transcription factor can be an effective anti-cancer agent when delivered using a targeted nanotherapy approach.
INTRODUCTION

Multiple myeloma (MM) is a malignancy derived from a clone of plasma cells, the terminally differentiated B-lymphocytes responsible for antibody production. MM is the second most common hematologic malignancy in the United States, and accounts for 1% of cancer deaths. Despite recent advances, the 5-year survival rate in patients with MM is less than 40% (1). While MM responds initially well to several classes of chemotherapy, (e.g. proteasome inhibitors, immunomodulatory drugs (IMiDs), and alkylating agents), virtually all patients eventually relapse and die from progressive disease.

The b-HLHZIP transcription factor c-Myc (MYC) has long been known to be a powerful oncogene activated in many types of cancer, and is a central driver of myeloma development (2). The expression of MYC increases with disease stage in MM, and MYC up-regulation may play a central role in the evolution of MGUS into MM (3). MYC activation is likely to be an early event in myeloma pathogenesis with MYC rearrangement present in about 15% of newly diagnosed myeloma (4) (1), which may be an underestimation of the prevalence of MYC translocations (5). Further, a transgenic mouse model of myeloma with targeted activation of MYC in germinal center B-cells further supports the role of MYC in MM (6).

Transcription factor’s relative position downstream as integrators of multiple signaling cascades makes them an attractive therapeutic target. Strategies for inhibition of MYC function include the anti-sense strategies (7), RNA interference (8), and interference with MYC-MAX dimerization using small molecules (9). Transformation by MYC is dependent upon dimerization with the bHLHZIP protein MAX, since MYC-MAX heterodimers are required for binding of MYC to E-Boxes in the vicinity of target genes (10) to regulate their expression, and modulate numerous biological functions (11) (12) (13). However, MYC remains a challenging target due to the difficulty of inhibiting protein-protein or protein-DNA interactions with small molecules (14) (15) (16) (17). After years of effort, several small-molecule inhibitors of the MYC-MAX interaction were reported (18), (9) (19) (20, 21), but development of these compounds has
been slowed due to rapid metabolism, poor bioavailability or inability of the drug to reach inhibitory concentrations in tumors (21). Yet, inhibitors of MYC function might be an effective and powerful therapeutic strategy if these hurdles could be overcome.

We have reported a nanotherapeutic drug delivery approach, termed “contact facilitated drug delivery” (CFDD) (22), that transfers nanoparticle lipid surfactant components to the targeted cell membrane through a hemifusion complexation process (23). Moreover, we have advanced this technology through the recent development of phospholipid Sn 2 prodrugs that stabilize and sequester the drug in the hydrophobic aspect of the outer lipid membrane of nanocolloids and prevent premature drug escape or metabolism during circulation to target cells (24, 25). Following transfer of the lipid monolayer components to the target cell membrane, cytosolic lipases enzymatically cleave the Sn 2 ester and liberate the drug into the cytosol (25, 26).

The overarching objective of this project was to characterize and demonstrate an integrin-targeted nanotherapy approach that would improve the efficacy of a potent small molecule inhibitor of MYC-MAX dimerization to increase survival in MM. Specific goals were to: 1) characterize the relative effectiveness and bioavailability of a candidate Sn 2 lipase labile MYC-MAX antagonist prodrug (MI1-PD) versus free compound (MI1) in myeloma cells, 2) demonstrate the efficacy of the MI1-PD incorporated into the integrin-targeted nanoparticles, αvβ3 versus VLA-4, in human MM cell lines and, and 3) assess the survival efficacy of targeted MI1-PD nanoparticles in a metastatic model of MM in mice.
MATERIALS AND METHODS

Cell lines

Cells were cultured in optimized culture media: H929 and U266 (purchased from ATCC in 2003 and frozen and stocked cells without passage were thawed in January 2012 for this project that were not tested in our place) LP1, UTMC2 and KMS11 (a generous gift from Dr. P. Leif Bergsagel, Mayo Clinic, Scottsdale, AZ) and 5TGM1 myeloma cells (a generous gift from Dr. G. Mundy, University of Texas, San Antonio, TX). All cells were grown in RPMI 1640 (Cambrex Bio Science Walkersville Inc., Walkersville, MD) and fetal calf serum (FCS; HyClone, Logan, UT) (10%) plus penicillin-streptomycin (P/S, 1%). All cell lines were cultured at 37°C and 5% CO2. Cells were incubated for 1h with 1mM MnCl₂ prior to all treatments.

Myc inhibitor (MI1) and prodrug (MI1-PD)

The synthesis involved alterations to the index compound (10058-F4) to introduce a short piperidine amine moiety for functionalization as the Sn 2 prodrug (20). (Figure 1A, Supplemental Data: Figures 1) Myc-inhibitor-1 prodrug (MI1-PD) was synthesized in three steps. Briefly, 4-ethyl benzaldehyde underwent aldol condensation reaction with rhodanine in the presence of a catalytic amount of Tween 80 in potassium carbonate solution at ambient temperature. The mixture was neutralized with 5% HCl and the precipitant treated with saturated sodium hydrogen sulfite (NaHSO₃), which was re-crystallized with aqueous ethanol to produce a bright yellow colored rhodanine derivative in 72% yield (HRMS calcd. 249, found MH⁺ 250 Da). This compound was reacted with mono-boc (tert-butoxycarbonyl) and formaldehyde. Deprotection of the Boc group in presence of 1:1 HCl in dioxane afforded the Myc-inhibitor-1 (5, HRMS calcd. 375, found MH⁺ 376 Da). Finally, the Myc-inhibitor-1 (MI1) was esterified with 1-palmitoyl-2-azelaoyl PC (fatty acid modified oxidized lipid 16:0-9:0 COOH PC; PAzPC) using a dicyclohexyl carbodiimide (DCC)/ 4-dimethyl amino pyridine (DMAP) mediated coupling to produce Myc-rhodanine prodrug (MI1-PD) in 43% yield.
**αβ₃---Integrin homing ligand**

The αβ₃-integrin antagonist was a quinalone nonpeptide developed by Bristol-Myers Squibb Medical Imaging (US patent 6,511,648 and related patents), which was initially reported and characterized as the $^{111}$In-DOTA conjugate RP748 and cyan 5.5 homologue, TA145. (27). The specificity of the αβ₃-ligand mirrors that of LM609 and has a 15-fold preference for the Mn²⁺ activated receptor (28) The IC₅₀ estimates for αβ₃α₅β₅ and GP IIbIIIa were >10 µM (BMSMI, Billerica, MA, USA, US patent 6,511,648 and related patents). The antagonist was a gift from Kereos, Inc. (Figure 1A)

**VLA-4 homing Ligand**

The VLA-4 ligand was modified from Peng et al. (29) Briefly, ring amide 4-methylbenzhydrylamine resin (MBHA) was fluorenlymethylloxycarbonyl chloride (Fmoc) deprotected with 20% piperidine in dimethylformamide (DMF). Fmoc-Ach-OH dissolved in hydroxy-benzotriazole (HObt) and 1,3-diisopropylcarbodiimide (DIC) in DMF was coupled at RT for 2h. The Fmoc deprotection with 20% piperidine, and serial coupling and deprotection cycles with Fmoc-Aad(tBu) and Fmoc- Lys(Dde) were performed. After removal of Fmoc, a solution of 2-(4-(3-o-tolylureido)phenyl)acetic acid, HOBt and DIC in DMF was added overnight, washed, and the Dde protecting group removed with 2% hydrazine in DMF. A solution of trans-3-(3-pyridyl) acrylic acid, HOBt and DIC in DMF was added and coupling ensured via a negative Kaiser test. The crude VLA-4 product was cleaved with 95% trifluoroacetate (TFA): 2.5% water: 2.5% triisopropylsilane, precipitated with diethyl ether and purified using RP-HPLC. (Figure 1A). VLA-4 ligand dissolved in ethanol was mixed with 2-iminothiolane in methanol and allowed to react for 2h @ 25 C. N-(4-(P-maleimidophenyl) butyrl)-phophatidylethanolamine MPB-PEG-DSPE (Avanti-Polar Lipid, Alabaster, AL). The purified lyophilized sample was a white solid (VLA-4-PEG-DSPE).
**Nanoparticle Synthesis**

Nanoparticles were prepared as a microfluidized suspension of 20% (v/v). Perfluorocarbon (PFC) nanoparticles combined perfluorooctyl bromide (PFOB) (Exflour Inc., Round Rock, TX) with a 2.0% (w/v) of a surfactant co-mixture, and 1.7% (w/v) glycerin in pH 6.5 carbonate buffer. Polysorbate micelles utilized sorbitan sesquioleate (Sigma Aldrich, Inc., MO) in place of the PFC. The surfactant co-mixtures included 2 mole% of MI1-PD, 0.15 mole% of α₃β₃-ligand or VLA-4-ligand conjugated lipids, and ~96.5 mole% of lecithin. Non-targeted nanoparticles excluded the homing ligands and no-drug nanoparticles excluded the MI1-PD. Rhodamine-conjugated to phosphatidylethanolamine (0.2 mole%) was incorporated into the lipid surfactant for flow cytometry. The surfactant components for each formulation were combined with the perfluorocarbon or polysorbate, buffer, and glycerin mixtures and were homogenized at 20,000 psi for 4 minutes at 4°C with a microfluidics (M110s, Microfluidics, Inc). The nanoparticles were preserved under inert gas in sterile sealed vials at 4°C until use. Hydrodynamic particle sizes for the MI1-PD incorporated PFC nanoparticles and polysorbate micelles were 210±32 nm and 16±4 nm, respectively, with a narrow distribution (polydispersity indexes, PDI: ~0.1-0.2). The negative electrophoretic potentials (ca.-20±6 mV) point to the colloidal stability and successful lipid encapsulation. Further nanoparticle characterizations, pharmacokinetic profiles and organ biodistributions were reported (30). *(Figure 1B, See Supplemental Data: Figures 2 and 3).*

**Immunoblot analysis**

Western blots were performed for integrin αᵥ and α₄ as described previously (31). Integrin αᵥ (Cell signaling) and α₄ (Santa Cruz Biotechnology, I NC) anti-β-actin (Sigma, St. Louis, MO) as primary antibodies. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences Corp., Piscataway, NJ) and visualized by enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL).
Flow cytometry using antibodies and fluorescent nanoparticles

Flow cytometry was performed on the human in H929, U266 and 5TG1 mouse MM cells as described previously (32). Apoptosis was analyzed using Annexin V. Anti-human integrin αvβ3 antibody, clone LM609 (Millipore Corporation, Billerica, MA, USA), anti-mouse integrin α4 and β1 (eBiosciences, San Diego, CA) and Rhodamine-labeled NPs were used for flow cytometry (FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, California, USA). Analysis was performed using FLOJO software (Tree Star, San Carlos, CA).

MM Cell Viability Assay

MTT (Sigma-Aldrich) was used for cell viability, as previously described (33). A total of 5,000 cells per well were plated in 96-well plates with indicated concentrations of drugs. After 24h, 10 μl MTT (Sigma-Aldrich) was added and HCl/ isopropanol was added after 4h. Absorbance was measured at 570 and 630 nm using a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA).

Detection of apoptosis

Apoptosis was analysed on cells treated with 50μM of MI1 and MI1-PD at 24h using the Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturers instructions.

Studies in the mouse model for myeloma

A KaLwRij Myeloma mouse model was used as described in (32). KaLwRij mice were inoculated intravenously with 1 × 10^6 5TG1 cells and distributed into six groups of 6–8 mice, and treated intravenously (a) non-targeted (NT) or targeted (T), (b) drug-bearing (D) or no drug (ND) PFC or micelle NPs. The following treatment cohorts were studied 1) ND/NT 200nm PFC NP; 2) T/ND 200 nm PFC NP; 3) T/D 200nm PFC NP; 4) NT/ND 20nm micelles; 5) T/ND 20 nm
micelles, and 6) T/D 20 nm micelles. 5TGM1 cells were injected via tail vein on Day 0. NPs were administered by tail vein injection on days 3, 5, 7, 10, 12, and 14 with 50μl of (MI1, 0.145 mg/ml) per mouse. Sera were collected on day 17, diluted 1:2 in PBS and analyzed by serum protein electrophoresis (SPEP) on a QuickGel Chamber apparatus using pre-casted QuickGels (Helena Laboratories) according to manufacturer’s instruction. Densitometric analysis of the SPEP traces was performed using the clinically certified Helena QuickScan 2000 workstation, allowing a precise quantification of the various serum fractions, including the measurements of gamma/albumin ratio.

**Statistical Analysis**

All statistical tests were carried out using GraphPad Prism™ software (GraphPad™, San Diego, USA). Statistical comparisons were performed with ANOVA and unpaired t-test for the comparison of in vitro drug treatments and SPEPs. Mantel-Cox test was used for mouse survival curves. Quantitative data are presented as the mean ± standard error of the mean (SEM) with p<0.05 considered significant.

**RESULTS**

*Bioactivity of the Sn-2 Prodrug (MI1-PD) and the parent drug (MI1) in vitro.*

The cytotoxic activities of the base compound, MI1, and the prodrug MI1-PD were evaluated and compared in human (H929 and U266) and mouse (5TGM1) multiple myeloma cell lines at concentrations ranging from 1.0 nM to 100 μM. MTT cell viability assay showed that MI1-PD decreased cell viability more (p<0.05) than MI1 on an equimolar basis (Figure 2A). MI1-PD induced significant apoptosis in the three MM cell lines, 92%, 91% and 91% of control, respectively. MI1 also increased (p<0.05) the amount of apoptosis in these cells, but the magnitude of response was significantly less, 19%, 31% and 31%, respectively, than that observed with MI1-PD (p<0.05) (Figure 2B).
Expression of α₃β₃-integrin by multiple myeloma cell lines

Nanotherapeutic delivery of MI1-PD is dependent upon the specific binding of nanoparticle to target cells, which then allows the formation of a membrane hemifusion complex to afford prodrug transfer. The suitability of using α₃β₃-integrin as a MM target was assessed by Western blot analysis evaluating the relative expression of the biomarker across a series of human MM cell lines. α₃-integrin was abundant in H929 and U266 cells and less so in UTMC2 cells. In contrast, little or no α₃-integrin was detected with LP1 or KMS11 MM cell lines (Figure 3A). α₃β₃-integrin surface expression was correlated with expression assessed in cell lysates (Figure 3B). LP1 cells expressed α₃β₃-integrin at very low levels (< 5%). The binding of rhodamine-labeled nanoparticles with and without α₃β₃-integrin targeting were compared using human MM cell lines (Figure 3B). Non-targeted NP had very low nonspecific adherence to any MM cells. However, α₃β₃-targeted NP had high binding to the H929 (64%) and moderate binding to U266 (26%) MM cells (Figure 3B). Binding of the α₃β₃-targeted NP to the LP1 cell line was negligible and no different than the non-targeted control. In anticipation of using these NPs in a mouse model of MM, the 5TGM1 murine cell line was evaluated for α₃β₃-integrin expression, and was found to express this integrin present at very low levels (8.1%, data not shown).

Expression of VLA-4 by human multiple myeloma cells

The α₄-integrin component of the VLA-4 heterodimer was strongly expressed by all human MM cell types except LP1, which was only slightly positive (Figure 3A). Replication of the nonspecific binding of non-targeted NP to MM cells yielded a negative result similar to the previous characterization experiment studying the α₃β₃-integrin. Rhodamine-labeled VLA-4 targeted NP bound to a large percentage of the human cell lines except LP1. As with α₃β₃-integrin, the highest surface expression rates for VLA-4 were noted for the H929 and U266 cells. KMS11 and UTMC2 cells, which had low or negligible α₃β₃ NP adherence, bound the
VLA-4 NP to a greater degree (Figure 3B). 5TGM1 expressed high levels of integrin \( \beta_1 \) (86.6%), \( \alpha_4 \) (90.8%) and also bound the VLA-4 NP (78.5%). (Figure 3C)

**Nanoparticle delivery and response to the MI1-PD in myeloma cell lines.**

Next, four groups of NP with and without peptidomimetic homing ligands, and with and without MI1-PD prodrug were compared for integrin targeting and drug delivery effectiveness. The dependence of NP targeting on the delivery and efficacy of MI1-PD, on an equivalent molar dosage basis was evaluated in H929, U266 and KMS11 human and 5TGM1 mouse myeloma cell lines. As a control, free MI1-PD in DMSO potently decreased cell proliferation in all MM cell lines relative to the media or DMSO alone (p<0.05). Free MI1 had lower potency, and was less cytotoxic compared to media only or DMSO only (p>0.05, Figure 4). MI1-PD alone was highly cytotoxic. We hypothesized, encapsulating MI1-PD within a ligand-targeted lipid-based nanosystem would enhance MI1 delivery with the prodrug form by preventing premature losses during systemic circulation and by protecting the compound within the hydrophobic membrane from plasma enzymatic degradation and hydrolysis, which have been previous barriers to the success of the cMyc-inhibitors. Of note, drug-bearing non-targeted NP had negligible cytotoxicity in the MM cell lines tested. Likewise, MM cells exposed to drug-free NP, whether targeted to VLA-4 or the \( \alpha_\nu\beta_3 \)-integrin, had no significant impact on cell viability compared to the media or DMSO controls (p<0.05, Figure 4). Cell proliferation of the H929 (Figure 4A) and U266 cells (Figure 4B), which highly expressed both the \( \alpha_\nu\beta_3 \) and VLA-4 integrin biomarkers, was reduced (p<0.05) equivalently by NPs targeted to either receptor. Only the NPs targeted to VLA-4 were effective in decreasing the cell viability in the human KMS11 cells, which appreciably expressed VLA-4 and minimal \( \alpha_\nu\beta_3 \)-integrin. (Figure 4C) This cell line illustrates the dependence and specificity of NP targeting for efficacious drug delivery. For mouse 5TGM1 cells, which were strongly positive for VLA-4 expression, VLA-4 NPs incorporating MI1-PD
effectively decreased cell viability (p<0.05). In cells with low α₃β₃-integrin expression, α₃β₃-targeted MI1-PD NPs had little impact on cell viability (Figure 4D).

**Anti-MYC nanoparticles prolong survival in a mouse model of MM**

The therapeutic potential of VLA-4-targeted MI1-PD-containing NP were evaluated in a preclinical metastatic model of MM. KaLwRij mice were inoculated intravenously with 1 × 10⁶ 5TGM1 cells and mice were distributed into six groups of 6–8 mice, and were treated intravenously with: 1) ND/NT 200; 2) T/ND 200; 3) T/D 200; 4) NT/ND 20; 5) T/ND 20 and 6) T/D 20. (Figure 5B) on days 3, 5, 7, 10, 12, and 14. MI1 and MI1-PD alone without NPs had no survival benefit for 5TGM1/KaLwRij myeloma mice versus DMSO alone. (Supplemental Data: Figure 4). Disease progression was monitored by survival and serial measurements of serum paraprotein on day 17. The tumor burden as measured by serum immunoglobulin was decreased (p<0.05) in mice treated with targeted MI1-PD NP as compared to NT/ND NPs (Figure 5C & 5D). In these mice, administration of VLA-4-targeted MI1-PD NPs conferred significant survival benefits compared to respective NP controls (T/D 200: 46 days vs. 28 days, p<0.05) and T/D 20: 52 days vs. 29 days, p=0.001, Figures 5E & F. These data demonstrate the efficacy of VLA-4-targeted anti-MYC NPs in a preclinical model of MM with the smaller 20nm micellar NP (T/D 20) being more effective than the 200nm PFC NP. Of note, these data are the first in vivo results utilizing MI1, MI1-PD alone or incorporated into lipid based particles, moreover, they represent the initial in vivo use of the 20 nM micelles for targeted drug delivery.
DISCUSSION

Development of clinically useful inhibitors of commonly overexpressed MYC oncoprotein has been a long-sought goal. This project demonstrates the pre-clinical efficacy of a ligand-directed nanomedicine approach to target a lipase-labile c-MYC-MAX inhibitor, MI1-PD, to protect the drug from systemic degradation and to improve its bioavailability. Delivery of the drug (MI1-PD via a CFDD transfer mechanism promoted by the binding of the nanoparticle through integrin targeting to MM cells provided effective drug delivery into the target cells in which cytosolic lipases liberated the active compound into the cytosol to exert its biological effect.

In vivo studies of the VLA-4 targeted MI1-PD NPs employed the orthotopic 5TGM1 mouse model of MM, since 5TGM1 cells expressed VLA-4 robustly. In vivo results for VLA-4 targeted MI1-PD NPs regardless of size recapitulated the in vitro findings. VLA-targeted MI1-PD NP conferred significant survival benefit in mice versus control NPs, with 20nm NPs having a higher survival benefit than the 200nm NPs (p<0.05). The number of mice surviving in the 20nm cohort was higher than the 200nm in any given week, which was further corroborated by the quantification of the gamma peak in the two cohorts. Neither MI1 nor MI1-PD provided survival benefit when administered systemically as free compounds. These results support the contention that the VLA-4 targeted NPs concentrate drug in the tumor and within the cells by overcoming prior issues of rapid systemic degradation and ultimately inadequate MM intracellular bioavailability.

In vitro, MI1-PD in DMSO was more effective in MM cells, than equimolar dosages of the free drug analogue, MI1. This improved potency likely reflected improved cellular bioavailability of the phospholipid prodrug. In addition, the distribution of MI1-PD throughout the inner cell membranes may create a drug depot from which the active compound could be liberated over a longer timeframe. Across the range of drug exposure concentrations tested, both compounds decreased cell-viability in a parallel monotonic fashion with no evidence of plateau effect noted,
indicating that neither intracellular drug release nor transcription factor pathway inhibition were limiting mechanisms. Interestingly, U266 cells, which have low c-MYC expression, (3) were sensitive to MI1-PD and MI1, at higher molar exposures, corroborating reports that 10058-F4 can inhibit n-MYC (34, 35).

Integrins VLA-4 and α_vβ3-integrins are critical for adhesion of MM cells to bone marrow stromal cells and both integrins have been suggested as therapeutic targets for MM treatment. (36) Based on the pattern of expression of activated α_vβ3 and VLA-4 integrins in MM cell lines (Figure 3), either integrin might be used as a preferential biomarker of human MM, but VLA-4 was expressed more widely in the human cell lines. Of the human myeloma cells evaluated, all but one expressed VLA-4. One myeloma cell line, LP1, expressed neither α_vβ3-integrin nor VLA-4 at significant levels and it did not show any cytotoxic response when treated with either of the two integrin-targeted MI1-PD nanoparticles. The murine 5TGM1 cells expressed high levels of VLA-4 and poorly expressed the α_vβ3 integrin, and appropriately, VLA-4-targeted MI1-PD nanoparticles were significantly more effective than those targeted to α_vβ3 in vitro (Figure 3). Indeed, others have targeted this integrin with liposomal doxorubicin in preclinical mouse models. (37)

While the cellular uptake of free MI1 or MI1-PD was dependent on the concentration of drug exposure in the cell medium, for the targeted nanoparticles the efficacy of MI1-PD delivered to the myeloma cells was dictated by the target integrin expression level. The non-targeted MI1-PD nanoparticles elicited no significant effects. Two of the human myeloma cell lines, H929 and U266 expressed both biosignatures abundantly and showed effective cytotoxicity when treated with either integrin-targeted MI1-PD nanoparticle. Presumably, high expression of the integrin on the target cells accommodated larger number integrin targeted nanoparticles, thereby affording higher intracellular drug levels. Given the heterogeneity of VLA-4 and α_vβ3-integrin expression and likely other biomarkers, a personalized medicine approach founded on prescreening results of myeloma cell biomarker expressions may enhance clinical management.
For human myelomas with negligible expression of VLA-4 and αβ3-integrin, as noted for the LP1 cells, further investigation of myeloma cell surface markers will be required to identify alternative targets with adequate expression levels.

In summary, we have developed a nanomedicine therapeutic approach to MM using a lipase-labile MYC-MAX inhibitor prodrug carried by integrin-targeted nanoparticles and for the first time showed reduced tumor burden in live mice. The Sn 2 phospholipid prodrug incorporates an analog of known MYC-MAX dimerization inhibitor. The intracellular lipase-labile prodrug, MI1-PD, had significantly increased potency against myeloma cells in culture compared to its free drug counterpart, most likely due to increased cell bioavailability. When incorporated into lipid surfactant-coated nanoparticles and targeted to VLA-4 or the αβ3-integrin, the MI1-PD was effective against cell lines expressing the biomarker strongly, and less potent when receptor expression was low or absent. In a mouse model of disseminated myeloma, administration of VLA-4 targeted MI1-PD NP conferred significant survival benefit versus non-targeted and drug-free controls. Neither MI1 nor MI1-PD provided survival benefit when administered systemically as free compounds. These data provide a strong foundation to reconsider transcription factor antagonism as a viable therapeutic strategy and in particular further pursue anti-MYC nanomedicine approaches to myeloma and perhaps other cancers.
REFERENCES


FIGURE LEGENDS

Figure 1: Structures and mechanism of drug delivery. A) Chemical structures of VLA-4-PEG-phospholipid conjugate, αvβ3-PEG-phospholipid conjugate and MI1-PD. B) Schematic representation illustrating nanoparticle (NP) design and lipid monolayer-mediated intracellular release of MYC-inhibitor from MI1-PD. The Myc prodrug (MI1-PD) from Panel A is represented by circles. The integrin targeting ligands (VLA-4-PEG-phospholipid conjugate and αvβ3-PEG-phospholipid conjugate) from Panel A is represented by triangles. (a) Anti-Myc NPs are targeted by the homing ligand to myeloma (MM) cells expressing integrin proteins on their cell surface (represented by V). (b) Proximity induces hemi-fusion of lipid layers between NP and MM cells. (c) The MI1-PD in the NP membrane is then exposed to cellular lipases that (d) cleave the phospholipid moiety of the prodrug and release the anti-Myc moiety into the MM cell.

Figure 2: Myc inhibitor 1 (MI1) and MI1 prodrug (MI1-PD) decrease viability and induce apoptosis in myeloma cell lines. A) MTT assay for cell viability at 24h with MI1 and MI1-PD at given concentrations in H929, U266 and 5TGM1 cells. Values are average of three separate experiments in triplicates and expressed as mean ± SD. *p < 0.05. B) Representative histograms of Annexin V-PE staining of cells for apoptosis following MI1 and MI1-PD treatment (left and right columns respectively) at given concentrations in H929, U266 and 5TGM1 cells at 24h.

Figure 3: Total and surface expression of integrin αvβ3 and VLA-4 proteins on human MM cells. Representative Western blots with total protein from human MM cell lines for endogenous levels of Integrins A) Immunoblots for Integrins αv and α4 and loading control β-actin are also shown as the loading controls. Each blot is representative of a set of three separate experiments yielding similar results. Representative histograms showing integrin by flow cytometry, each histogram is representative of a set of three separate experiments yielding similar results for all the experiments: B) (i) Cell surface expression on integrin αvβ3 on given human MM cells by the binding to LM609-FITC. The non-activated MM cells are shown in the
area under grey panel and Mn$^{2+}$ activated under black panel. (ii) binding to rhodamine-labeled $\alpha_\nu\beta_3$-targeted and non-targeted MI1 PD NPs in Mn$^{2+}$ activated human MM cell lines. The cells binding to the non-targeted MI1-PD NPs are shown in the area under grey panel and $\alpha_\nu\beta_3$-targeted under black panel. (iii) binding to rhodamine-labeled VLA-4 targeted and non-targeted MI1 PD NPs in Mn$^{2+}$ activated human MM cell lines. The cells binding to the non-targeted MI1-PD NPs are shown in the area under grey panel and VLA-4-targeted under black panel. C) integrin $\beta_1$, Integrin $\alpha_4$ and rhodamine-labeled VLA-4-targeted and non-targeted MI1 PD NPs in Mn$^{2+}$ activated mouse 5TGM1 cell line. The cells binding nonspecifically are shown in the area under grey panel and positive staining under black panel.

**Figure 4:** Efficacy of the integrin-targeted anti-MYC NPs is dependent on respective expression of $\alpha_\nu\beta_3$ and VLA-4. MTT studies with media, DMSO MI1, MI1-PD, non-targeted, integrin $\alpha_\nu\beta_3$ and $\alpha_4\beta_1$ targeted MI1-PD NPs in (A) H929, (B) U266, (C) KMS11 and (D) 5TGM1 cells after 24h. Bars represent mean ± standard error of the mean of experiments repeated in six replicates.

**Figure 5:** Anti-MYC NPs prolong survival in a mouse model of multiple myeloma. (A) MTT studies in 5TGM1 cells at 24 h (B) The experimental protocol for the in vivo tumor growth assay. The groups were as follows: 1) ND/NT 200; 2) T/ND 200; 3) T/D 200; 4) NT/ND 20; 5) T/ND 20 and 6) T/D 20 all injected on days 3, 5, 7, 10, 12 and 14 following the i.v. injections of 5TGM1 cells (Day 0) (C &D) SPEP quantification of each treatment groups 3d after the last injection was given (Each dot represents SPEP value for one mouse) (E&F) Kaplan-Meier survival curves of the treated mice with 20 and 200nm NPs. (ND=no drug, NT=non-targeted)
A. VLA-4-PEG-phospholipid conjugate

\[
\text{Structure of VLA-4-PEG-phospholipid conjugate}
\]

\[\alpha\beta_3\text{-PEG-phospholipid conjugate}\]

\[\text{MI1-PD}\]

B

\[\alpha\beta_3\text{ Ligand or VLA-4 Ligand}\]

\[\text{cMyc phospholipids prodrug}\]

\[\text{MM Cell}\]

\[\text{NP}\]

\[\text{Phospholipids}\]

\[\text{Active targeting}\]

\[\text{Hemi fusion of lipid layers}\]

\[\text{Delivery of drug}\]
**FIGURE 2**

A. **Cell viability**

- MI-1
- MI-1PD

Drug concentration (µM)

H929

U266

5TGM1

B. **Apoptosis**

H929

U266

5TGM1

Annexin V
FIGURE 3

A. Integrin αv

B. H929 U266 KMS11 UTMC2 LP1

B. H929 U266 KMS11 UTMC2 LP1

C. 5TGM1

β1

α4

VLA4 NP

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FIGURE 4

A. H929

B. U266

C. KMS11

D. 5TGM1
A. 5TGM1 viability

B. Tumor mice dosage

C. SPEP NP200

D. SPEP NP20

E. Survival NP200

F. Survival NP20
Molecular Cancer Therapeutics

Small molecule MYC inhibitor conjugated to integrin-targeted nanoparticles extends survival in a mouse model of disseminated multiple myeloma

Deepthi Soodgupta, Dipanjan Pan, Grace Cui, et al.

* Mol Cancer Ther * Published OnlineFirst March 30, 2015.

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